QP631.T69

Adams

STIC-ILL

From: Baskar, Padmavathi
Sent: Monday, June 30, 2003 11:46 AM
To: STIC-ILL
Subject: ~~09/300621~~ 300612

Toxicon: please send this article asap. Thanks

Toxicon 1994, 32: 1237-1249.

Padma Baskar
Art Unit 1645
Patent Examiner/Biotechnology
CM-1, 8E-13
703-308-8886

Pergamon

0041-0101(94)00061-1

Toxicon, Vol. 32, No. 10, pp. 1237–1249, 1994

0041-0101/94 $7.00 + 0.00

ISOLATION AND PARTIAL CHARACTERIZATION OF AN ANTI-BOTHROPIC COMPLEX FROM THE SERUM OF SOUTH AMERICAN DIDELPHIDAE

JONAS PERALES,[1,2]* HAITY MOUSSATCHÉ,[2] SERGIO MARANGONI,[3] BENEDITO OLIVEIRA[3] and GILBERTO B. DOMONT[4]

[1]Departamento de Bioquímica, Escuela de Medicina, Universidad Centro Occidental, Barquisimeto, Venezuela; [2]Departamento de Fisiologia e Farmacodinâmica, Fundação Oswaldo Cruz, Avenida Brasil 4365, Caixa Postal 926, CEP 21045-900, Rio de Janeiro, Brazil; [3]Departamento de Bioquímica, Instituto de Biologia, UNICAMP, 13081, Campinas, SP, Brazil; and [4]Departamento de Bioquímica, Instituto de Química, UFRJ, 21910 Rio de Janeiro, Brazil

(*Received* 7 *September* 1992; *accepted* 20 *April* 1994)

J. PERALES, H. MOUSSATCHÉ, S. MARANGONI, B. OLIVEIRA and G. B. DOMONT. Isolation and partial characterization of an anti-bothropic complex from the serum of South American Didelphidae. *Toxicon* **32**, 1237–1249, 1994.—An anti-bothropic fraction (ABF) with anti-*Bothrops jararaca* venom activity tested in mice was isolated from the serum of some South American Didelphidae (*Didelphis marsupialis*, *Philander opossum* and *Lutreolina crassicaudata*) by DEAE-Sephacel chromatography. ABF from *D. marsupialis* was shown to be 12 times more active in protection assays on a weight basis than the serum proteins. A similiar fraction obtained from *Metachirus nudicaudatum* serum was shown to be inactive. An anti-bothropic complex (ABC) was isolated from *D. marsupialis* ABF. HPLC gel permeation chromatography of ABC from *D. marsupialis* indicated the presence of a main peak with mol. wt of 84,000. SDS-PAGE of this ABC showed the presence of two subunits of 48,000 and 43,000. The active ABF isolated from *P. opossum* and *L. crassicaudata* also showed the presence of these subunits by SDS-PAGE. Isolation of the 48,000 mol. wt *D. marsupialis* subunit by HPLC-hydrophobic interaction chromatography demonstrated that the 43,000 subunit was essential for the protective action of the complex. Both subunits from *D. marsupialis*, *P. opossum* and *L. crassicaudata* were Western-blotted and N-terminal sequenced. No N-terminal amino acid was found for the 43,000 subunit, whereas for the 48,000 subunit a high degree of homology was found:

D. marsupialis: H_2N–L K A M D P T P P L W I K T E X P . ;

L. crassicaudata: H_2N–L K A M D P T P P L W I Q T E . . . ;

P. opossum: H_2N–L K A M D T T P E . . .

No significant homology with known proteins was detected.

* Author to whom correspondence should be addressed, at address[2].

INTRODUCTION

SOME animals are resistant to certain snake venoms. Several reports exist ab ut the resistance of venomous and non-venomous snakes to the toxic effects of snake venoms (CERDAS and LOMONTE, 1982; FONTANA, 1781; MIRANDA *et al.*, 1982; OVADIA *et al.*, 1975; PEREZ *et al.*, 1978*a*; PHILPOT and SMITH, 1950). The resistance to snake venoms is not an exclusive property of the snakes; it is also present in some mammals (MOUSSATCHÉ and LEONARDI, 1982; OVADIA and KOCHVA, 1977; PEREZ *et al.*, 1978*b*; VELLARD, 1945). In many cases, the resistance of these animals to snake venoms could be explained by the presence of neutralizing factors in their blood sera (FORTES-DIAS *et al.*, 1990; MOUSSATCHÉ and PERALES, 1989; OMORI-SATOH, 1977; OVADIA *et al.*, 1975; OVADIA, 1978; PERALES *et al.*, 1986). Several neutralizing proteins have been isolated and characterized from the serum of different animals. Examples are the antineurotoxic protein from *Vipera palaestinae* serum (OVADIA *et al.*, 1977) and the antihemorrhagic proteins isolated from sera of *Trimeresurus flavoviridis* (OMORI-SATOH *et al.*, 1972), *Vipera palaestinae* (OVADIA *et al.*, 1975, 1977), *Crotalus atrox* (WEISSENBERG *et al.*, 1991), *Bothrops jararaca* (TANIZAKI *et al.*, 1991), *Dinodon semicarinatus* (TOMIHARA *et al.*, 1988), *Didelphis marsupialis* (MOUSSATCHÉ *et al.*, 1978, 1979; PERALES *et al.*, 1986, 1989), *Didelphis virginiana* (MENCHACA and PEREZ, 1981), *Didelphis albiventris* (LANDUCCI *et al.*, 1990), *Neotoma micropus* (GARCIA and PEREZ, 1984), *Erinaceus europaeus* (DE WIT and WESTRÖM, 1987), *Herpestes edwardsii* (TOMIHARA *et al.*, 1987) and *Sigmodon hispidus* (PICHYANGKUL and PEREZ, 1981). The protective antihemorrhagic and antineurotoxic proteins have some common characteristics: they are acidic proteins with isoelectric points ranging between 4.0 and 5.4; their molecular masses vary from 52 to 90 kDa, with one exception being the antihemorrhagic factor from *Erinaceus europaeus* (539–780 kDa). None has proteolytic activity and they are not immunoglobulins (for review, see DOMONT *et al.*, 1991).

The objective of this paper is to describe the isolation, and some physico-chemical and structural properties, of a proteic complex with anti-*Bothrops jararaca* venom activity present in the serum of *Didelphis marsupialis* ('gamba', common South American opossum), *Philander opossum* ('cuica', gray four-eyed opossum) and *Lutreolina crassicaudata* ('cuica', brown four-eyed opossum) Didelphidae living in South America.

MATERIALS AND METHODS

Animals, serum and venom

Didelphis marsupialis, *Philander opossum*, *Lutreolina crassicaudata* and *Metachirus nudicaudatum* specimens were caught in the Rio de Janeiro area. They were fed and watered *ad libitum* for several weeks before bleeding by heart puncture. After blood clotting at room temperature, serum was separated by cold centrifugation and freeze-dried. In some experiments, centrifuged serum was dialyzed and directly processed. All experiments with animals were performed in accordance with the Ethical Standards provided by IST.

Bothrops jararaca venom was kindly supplied by Instituto Butantan (IB), São Paulo, Brazil, and by Fundação Instituto Ezequiel Dias (IED), Minas Gerais, Brazil.

LD_{50} *determination*

Toxicity of both *B. jararaca* venoms were tested by mouse bioassay. Their i.p. LD_{50} was calculated by injecting Swiss Webster mice (18–20 g) in four groups of six mice each. Venom solutions were prepared by dissolving an appropriate weighted amount in saline. A 1.1-fold dose interval was used and each dose never exceeded 100 μl. The i.p. LD_{50} was calculated by Th mpson's moving average interpolation method (THOMPSON and WEIL, 1952). Death was assessed 24 hr after injecti n. IED venom was only used in the determination f the total protection of mice afforded by the different sera. f r the determination of the effective dose (ED_{50}) f *D. marsupialis* serum and for the neutralizing potency of ABF isolated from *D. marsupialis* serum as reported in the Results (Protective

assay). IB venom was employed throughout the rest of this study. In any case, the venom used can be identified by its LD_{50} value.

Animal resistance

Animal resistance to *Bothrops jararaca* venom was determined by i.p. injection in different specimens of the same species of increasing LD_{50} venom doses determined in mice as described in this paper. The highest doses reported in results were the highest doses used and not the highest doses that each animal could resist. These doses were very different because the number of animals available for each species varied. The most common marsupial was *D. marsupialis* and the least common was *L. crassicaudata*.

Protective assay

Generally, the protective activity of the different sera and their fractions were determined by i.p. injecting Swiss-Webster mice (18–20 g) with 5 μg protein/g (2 LD_{50}) of *Bothrops jararaca* venom (IB) mixed with the appropriate amount of each sample (from 10 up to 300 μg protein/g) both dissolved in a total volume of 0.1 ml of saline solution. The sample to be assayed and venom were incubated at 37°C for 30 min in a water bath according to DOMONT *et al.* (1991). Venom alone was injected as positive control and saline and protective samples were also injected as negative controls. Groups of six animals were used and mice survival was recorded after 24 hr.

For *D. marsupialis*, the amount of serum proteins and of its ABF that neutralized 1 LD_{50} in the assay conditions described above were calculated according to THOMPSON and WEIL (1952). The amount of venom (IED) was kept constant at 2 LD_{50} (9.2 μg/g) and the amount of serum proteins varied from 100 μg/g using a 1.3-fold interval and that of ABF increased from 10.4 μg/g by a 1.4-fold interval. Five groups of six mice were used and survival was recorded after 24 hr.

Protein determination

Protein concentration was determined as described by LOWRY *et al.* (1951) with bovine serum albumin (Sigma, U.S.A.) as standard.

Isolation of protective anti-bothropic fractions (ABF)

Anti-bothropic fractions (ABF) were prepared according to a previously described method (PERALES *et al.*, 1989) using DEAE-Sephacel chromatography (Pharmacia, Sweden) and under the conditions reported in the legends for Fig. 1. Elution was performed using an LKB system (Sweden) composed of a fraction collector Ultrorac II, an Uvicord S detector and a peristaltic Microperpex S pump.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed on 7.5% or 12.6% acrylamide (Sigma, U.S.A.) separating gels with 4% acrylamide stacking gels using the method of LAEMMLI (1970). Proteins were stained by 0.1% Coomassie brilliant blue R-250 (Sigma, U.S.A.) dissolved in aqueous 45% (v/v) methanol/10% (v/v) acetic acid for 30 min and then destained in aqueous 45% (v/v) methanol/10% (v/v) acetic acid.

Western blotting

For amino acid sequencing, the proteins were transferred to polyvinyldifluoride membrane (Problott, Applied Biosystems, U.S.A.) in 25 mM Tris/10 mM Gly/0.5 mM DTT, pH 8.3. The blot was then stained with 0.1% Coomassie brilliant blue R-250 in aqueous 50% methanol, for 5 min. After drying, the desired band was excised and subjected to N-terminal sequence analysis.

HPLC-gel permeation chromatography

This was conducted on a LKB system composed of a 2151 variable wavelength detector, a 2152 HPLC controller and a 2150 HPLC pump. Fractionation was achieved on a TSK G 3000 (7.5 × 300 mm) column (LKB, Sweden) using pH 7.0, 0.15 phosphate buffer, 0.05% sodium azide, at a flow rate of 0.1 ml/min.

Molecular mass

This was estimated by SDS-PAGE (WEBER and OSBORN, 1969) and gel-permeation (ANDREWS, 1964) as described above, in comparison with reference proteins.

Hydrophobic interaction chromatography

This was performed using either a 7.5 × 75 mm TSK-5PW Spherogel (Beckman, U.S.A.) column in the LKB-HPLC system described, at a flow rate of 1 ml/min, r using a Phenyl-Sepharose (Pharmacia, Sweden) column (15 × 75 mm) eluted in the LKB-l w pressure system at a flow rate f 0.5 ml/min. In both cases, proteins were chromatographed using first 1.0 M, and then discontinuously 0.1 M ammonium acetate, both at pH 7.1.

N-terminal amino acid sequence

Sequence analysis was carried out on an Applied Biosystems 477-A protein Sequencer. The amino acid phenylthiohydantoin derivatives were determined on-line with the aid of an Applied Biosystems 120A PTH-analyser. Modified programs for blotted proteins and proline residues were used.

RESULTS

LD_{50} *estimation*

In the conditions of our assay the LD_{50} values obtained for *Bothrops jararaca* venom in mice were 2.5 μg/g body weight for the Instituto Butantan (IB) venom and 4.6 μg/g for the Instituto Ezequiel Dias batch (IED).

Animal resistance

The i.p. injection of *B. jararaca* venom (IB) demonstrated that some Didelphidae are resistant to this crotalic venom. Table 1 shows the results obtained using different number of LD_{50} doses as well as their respective values in mg of venom injected per kg of animal tested. Because this was a trial and error assay and considering that the number of specimens available varied, the number of LD_{50} injected was arbitrarily chosen. Thus, *Didelphis marsupialis* specimens survived to upper doses of 160 LD_{50} or 400 μg of bothropic venom/g; *Philander opossum* to 8 LD_{50} or 20 μg/g; and *Lutreolina crassicaudata* to 6 LD_{50} or 15 μg/g; *Metachirus nudicaudatum* did not show any resistance, dying after injection of 2 LD_{50} or 5 μg/g.

TABLE 1. RESISTANCE OF SOME *Didelphidae* TO *Bothrops jararaca* VENOM

Animals	Animals tested (*n*)	LD_{50} injected* (*n*)	Venom injected (mg/kg)	Survival after 24 hr
D. marsupialis	2	2	5	Alive
	1	8	20	Alive
	1	20	50	Alive
	1	40	100	Alive
	1	80	200	Alive
	1	160	400	Alive
P. opossum	3	2	5	Alive
	1	5 + 5†	12.5 + 12.5†	Alive‡
	1	8	20	Alive
L. crassicaudata	1	1.5	3.75	Alive
	2	2	5	Alive
	2	3	7.5	Alive
	1	6	15	Alive
M. nudicaudatum	2	2	5	Dead

*LD_{50} = 2.5 μg/g (IB) as determined in mice.
†5 LD_{50} at time zero + 5 LD_{50} after 24 hr.
‡Survival after 48 hr.

Protective assay

Total pr tecti n of mice against 2 LD_{50} (9.2 μg/g) f *B. jararaca* venom (IED) was btained with 290 μg of *D. marsupialis* serum protein per g f mice. Its ED_{50} (the amount of serum proteins th·at neutralized one of the two LD_{50} injected in the assay) calculated using the moving average interpolation method (THOMPSON and WEIL, 1952) averaged 162 ± 18 μg/g. When the neutralizing ability of ABF isolated from *D. marsupialis* was assayed using the same methodology, a mean of 13.25 ± 0.9 μg/g was obtained for the neutralization of 1 LD_{50}. Using the value obtained for *D. marsupialis* serum proteins it was determined that 300 μg of serum proteins from *P. opossum* and *L. crassicaudata* per g of mice were able to protect them against the lethal action of 2 LD_{50} (9.2 μg/g). *Metachirus nudicaudatum* serum proteins did not protect mice at this same concentration.

Isolation of protective anti-bothropic fractions (ABF)

Sera of *D. marsupialis*, *L. crassicaudata*, *P. opossum* and *M. nudicaudatum* fractionated by ion-exchange chromatography on DEAE-Sephacel (Fig. 1) showed profiles with most of the proteins concentrated on two peaks. For *D. marsupialis*, *L. crassicaudata* and *P. opossum* (Fig. 1A, B, C) the protective action against the bothropic venom was found

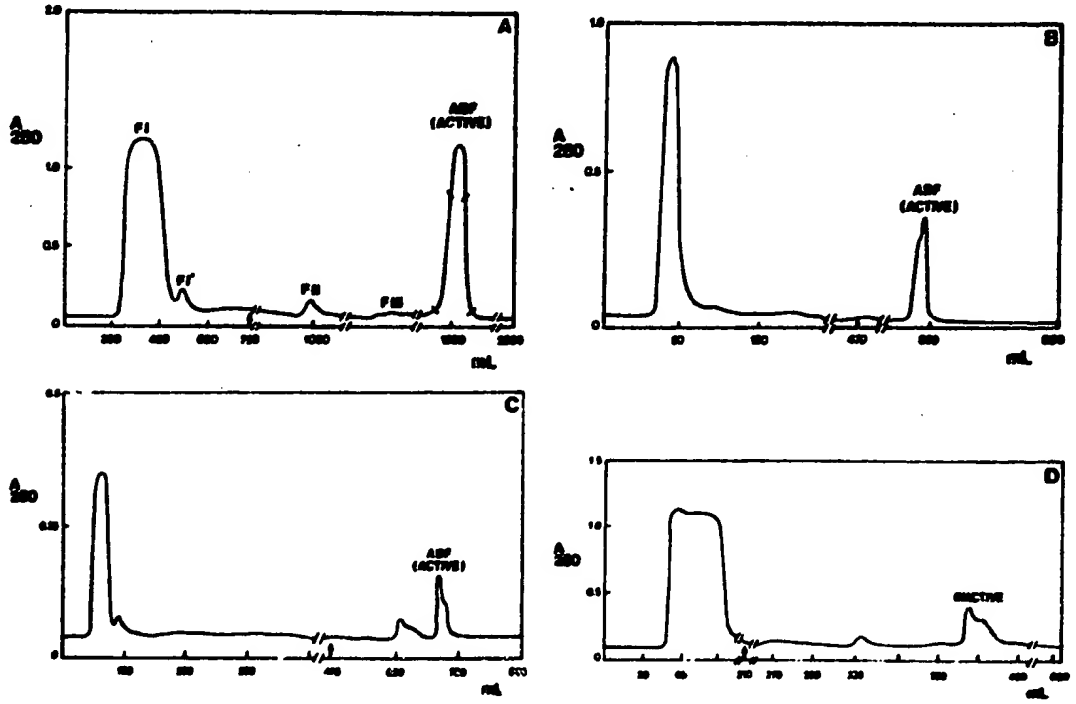

FIG. 1. DEAE-SEPHACEL EXCHANGE CHROMATOGRAPHY PROFILE OF *Didelphis marsupialis* (A), *Lutreolina crassicaudata* (B), *Philander opossum* (C) AND *Metachirus nudicaudatum* (D) SERUM. Sera were dialyzed for 24 hr at 4°C against 0.01 M, pH 3.7, sodium acetate. After centrifugation the supernatant was liyophilized or directly fractionated on a DEAE-Sephacel column, eluted initially with 0.01 M acetate buffer, pH 3.7, and finally with this same buffer containing 0.15 M sodium chloride at a flow rate f 0.5 ml/min. Ten milliliter aliquots were collected. Arrow signals buffer change. A: *Didelphis marsupialis* serum (4 g); 5 × 100 cm colum; bars limit pooled ascending, apex and descending porti ns f the active fraction; B: *Lutreolina crassicaudata* serum (4.3 ml); C: *Philander opossum* serum (4.6 ml); D: *Metachirus nudicaudatum* serum (6.0 ml): 2.5 × 21.5 cm column.

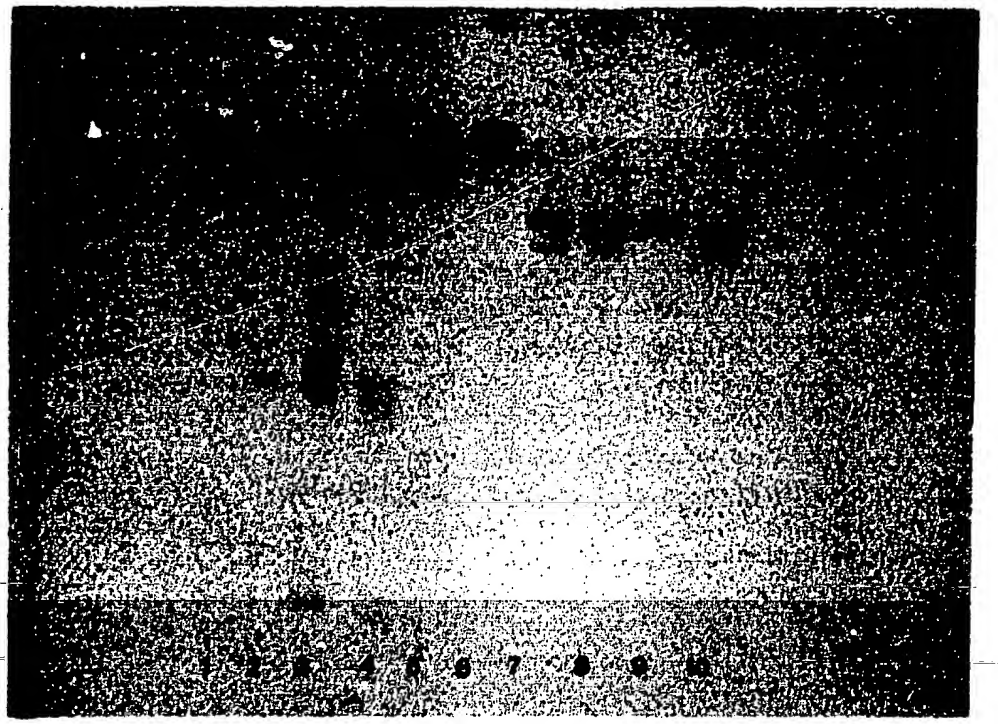

FIG. 2. SDS-PAGE (12.6%, LAEMMLI) OF FRACTIONS ISOLATED FROM *Didelphis marsupialis* SERUM BY DEAE-SEPHACEL CHROMATOGRAPHY WITH COOMASSIE BLUE STAIN.
Lane 1, Bovine serum albumin (66 kDa); lane 2, serum; lane 3, FI; lane 4, FII; lane 5, FIII; lanes 6–8, ascending, apex and descending regions of ABF peak, respectively; lane 9, pure 48 kDa subunit; 10: ovalbumin (45 kDa).

only in the last eluted peak, the one desorbed by sodium acetate buffer containing 0.15 M NaCl. Total protection was obtained using 30 μg/g of ABF as described in Methods. The equivalent last peak found in the serum of *M. nudicaudatum* (Fig. 1D), however, did not show protective activity using 50 μg/g. Figure 1A also marks the separated collection of the ascending, apex and descending portions of *D. marsupialis* anti-bothropic fraction.

SDS-polyacrylamide gel electrophoresis

The ascending, apex and descending portions of the anti-bothropic fraction from *D. marsupialis* were separately submitted to SDS-PAGE (Fig. 2). The descending side (lane 8) shows the presence of only two protein bands with estimated molecular masses of 48 and 43 kDa. The apex (lane 7) has a similar profile and includes the presence of two more faint bands: one near the position of bovine serum albumin and another in-between the two bands present in lane 8. Both the descending side and apex exerted protective action (10 μg/g) against *B. jararaca* venom. The ascending side (lane 6) has a different profile: it almost exclusively shows the presence of a strong protein band near bovine albumin and has no protective activity (50 μg/g). FI to FIII (lanes 3–5) are mixtures of several proteins; they do not have any of the protein bands present in lane 8 and did not show any protective activity either (150 μg/g).

Figure 3 compares the heterogeneity of the fractions obtained by 0.15 M NaCl desorpti n (ABF) from the DEAE-Sephacel column of each marsupial serum. Three of them, anti-bothropic fractions from *D. marsupialis* (lane 3), *P. opossum* (lane 4) and

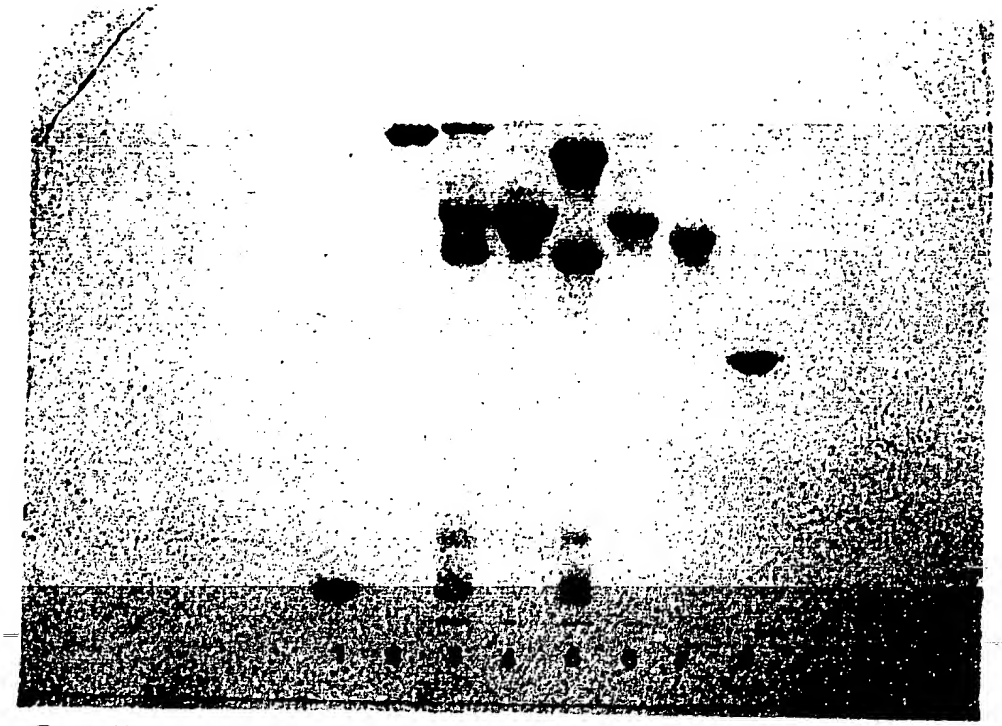

FIG. 3. SDS-PAGE (12.6%, LAEMMLI) OF FRACTIONS ISOLATED FROM DIFFERENT MARSUPIALS' SERA BY DEAE-SEPHACEL CHROMATOGRAPHY WITH COOMASSIE BLUE STAIN.
Lane 1, Alpha-lactalbumin (14.2 kDa); lane 2, bovine serum albumin (66 kDa); lane 3, ABF-*D. marsupialis*; lane 4, ABF-*P. opossum*; lane 5, ABF-*M. nudicaudatus*; lane 6, ABF-*L. crassicaudata*; lane 7, ovalbumin (45 kDa); lane 8, carbonic anhydrase (29 kDa).

L. crassicaudata (lane 6), have similar patterns near the position of ovalbumin; differences among them are found in band intensities near serum albumin (lane 7) and in the presence of fast migrating proteins (lanes 3 and 5). They all show the presence of the same two proteins bands present in the descending side of ABF isolated from *D. marsupialis*, and they all exerted protective action (30 μg/g) against *B. jararaca* venom. The equivalent sodium chloride desorbed peak obtained from *M. nudicaudatum* has a different SDS-PAGE profile (lane 5) and does not show the presence of these two proteins.

HPLC-gel permeation chromatography

The descending arm obtained from *D. marsupialis* serum by chromatography on a DEAE-Sephacel column was fractionated by molecular sieving. It showed a single peak by HPLC-gel permeation chromatography (Fig. 4A) with a calculated molecular mass of 84 kDa (Fig. 4B).

Hydrophobic interaction chromatography of ABF

The active fraction (ABF) obtained by DEAE-Sephacel chromatography from *D. marsupialis* serum was further fractionated using low-pressure and high-performance hydrophobic interaction chromatographies. Similar results were obtained f r the two systems (Fig. 5A, B). Figure 6 sh ws that F1P isolated by HPLC contains the 48 kDa subunit plus an additional band around 66 kDa (lane2); F1P obtained by the low-pressure

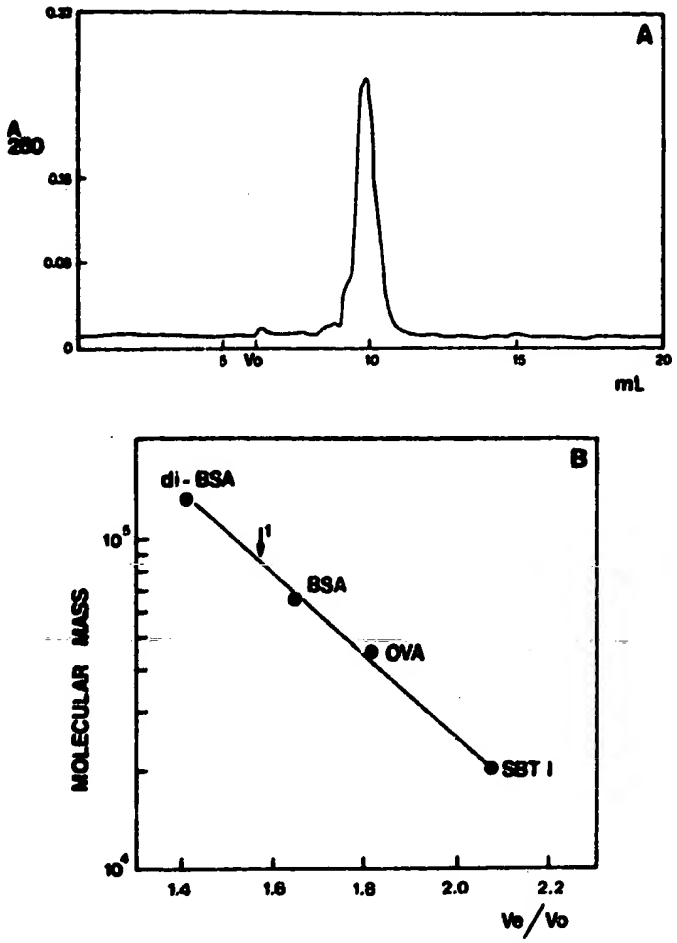

FIG. 4. (A) HPLC-GEL FILTRATION AND (B) MOLECULAR MASS DETERMINATION OF THE PROTECTIVE FRACTION ISOLATED FROM *Didelphis marsupialis*.
(A) Ten microliters of the descending side of ABF (40 μg/μl) obtained by DEAE-Sephacel chromatography from *Didelphis marsupialis* serum was applied to a 7.5 × 300 mm TSK G-3000 column (LKB) which was eluted with pH 7.0, 0.10 M phosphate buffer at a flow rate of 0.1 ml/min. (B) Molecular markers and the descending side of ABF from *D. marsupialis* serum were eluted as in A. Void volume (Blue dextran) of 5.90 ml; di-BSA, bovine serum albumin dimer (132 kDa); BSA, bovine serum albumin (66 kDa); OVA, ovalbumin (43 kDa); SBTI, soybean trypsin inhibitor (20.1 kDa). 1, Descending side of ABF from *D. marsupialis*.

system is the 48 kDa subunit devoid of any contaminants (lane 4). This same figure shows that F2P isolated by both techniques yielded the same heterogeneous profile as the impure active fraction (ABF) but enriched in the 43 kDa subunit (lanes 3 and 5).

When tested in protective assays, F1P (30 μg/g) did not protect mice from the lethality of *B. jararaca* venom. F2P (30 μg/g) prevented death when i.p. injected in a mixture with 5 μg/g (2 LD_{50}) of the venom but not when injected in lower doses (5, 10, and 20 μg/g).

N-terminal amino acid sequence

The 48 kDa fraction from *D. marsupialis* was isolated both by hydrophobic interaction chromat graphy and by Western blotting. Those from *P. opossum* and *L. crassicaudata* were electroblotted. The automatic Edman degradation of these proteins revealed that

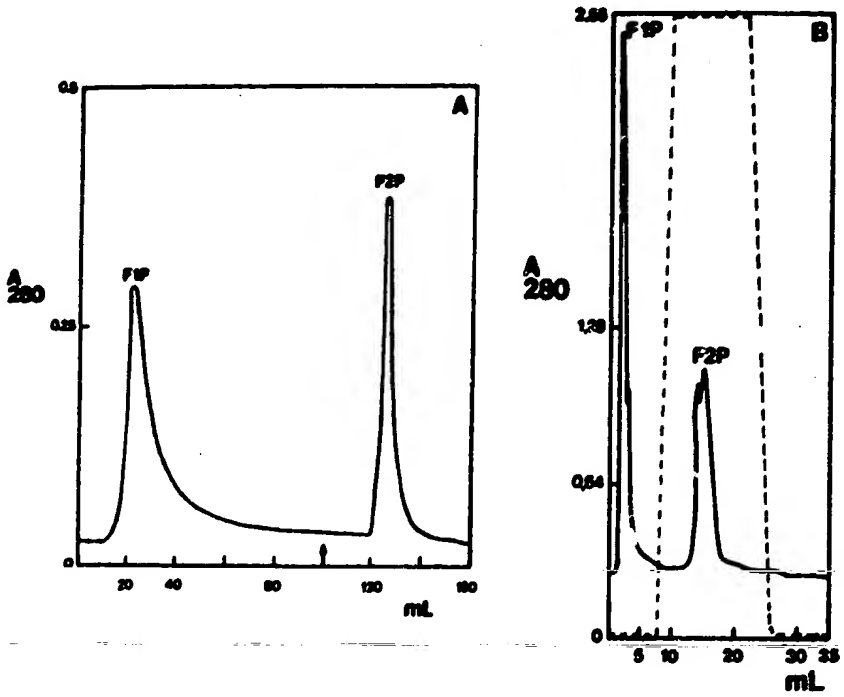

FIG. 5. (A) LOW-PRESSURE AND (B) HPLC-HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF ABF FROM *D. marsupialis*.

(A) Phenyl-Sepharose CL-4B hydrophobic chromatography profile of DEAE-rechromatographed ABF from *D. marsupialis* serum. Fifty milligrams of ABF dissolved in 2 ml of 1.0 M, pH 7.1, ammonium acetate were fractionated on a 7.5 cm height Phenyl-Sepharose column (Pharmacia K 16/20) first with the sample buffer (100 ml) and then (arrow) with 0.10 M ammonium acetate (60 ml) at a flow rate of 0.5 ml/min. Two milliliter aliquots were collected. (B) Hydrophobic interaction chromatography pattern of ABF isolated from *D. marsupialis* serum. ABF (5.4 mg) dissolved in 100 μl of 1.0 M, pH 7.1, ammonium acetate was fractionated on a 7.5 × 75 mm TSK-5PW Spherogel column. Elution was performed employing 1.0 M, pH 7.1 (A) and 0.10 M, pH 7.1 (B) ammonium acetate solutions at a flow rate of 1 ml/min: 7.5 min, 0% B; 10 min, 100% B; 21 min, 100% B; 26 min, 0% B and 35 min, 0% B. (——) Protein (- - -)% Buffer B).

their N-terminal sequence is highly homologous. The *D. marsupialis* subunit isolated by the two procedures showed the same amino acid sequence. The following N-terminal sequences were obtained:

	5				10				15					
D. marsupialis:	H_2N–L	K	A	M	D	P	T	P	P	L	W	I	K	T	E	X	P	. ;
L. crassicaudata:	H_2N–L	K	A	M	D	P	T	P	P	L	W	I	Q	T	E	.	.	. ;
P. opossum:	H_2N–L	K	A	M	D	T	T	P	E	.	.	.						

Attempts to sequence the 43 kDa subunits from the three resistant species were unsuccessful.

DISCUSSION

Natural immunity is a common property of various animals. Among the best known anti-venom proteins are antihemorrhagic and antineurotoxic factors found in the sera of snakes and mammals (for review see DOMONT *et al.*, 1991). The ability of the sera of

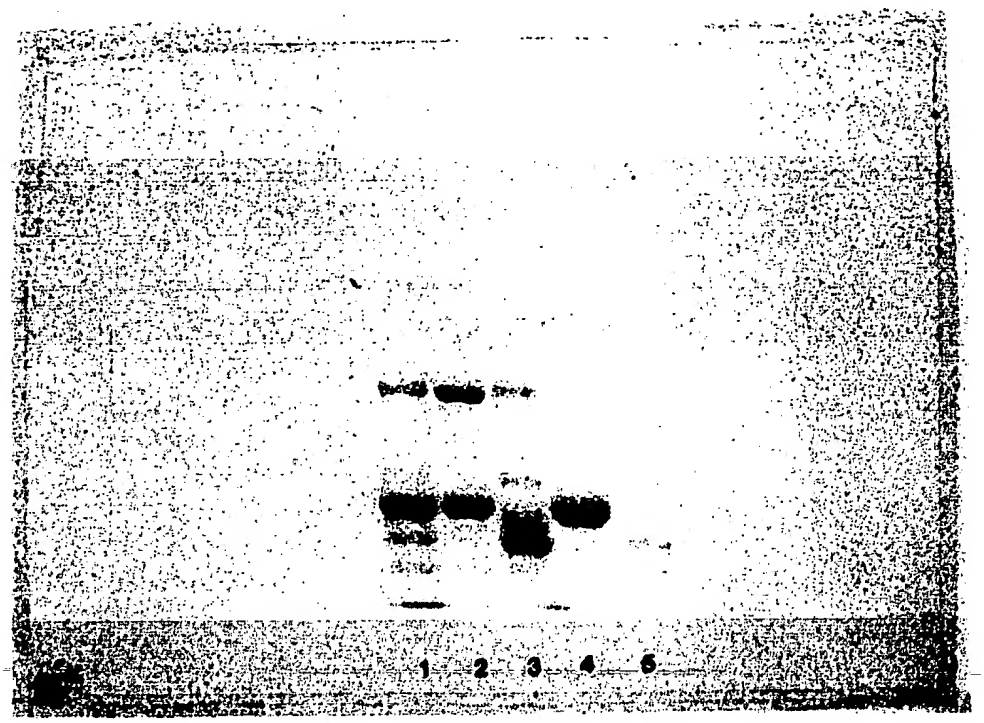

FIG. 6. SDS-PAGE (7.5%, LAEMMLI) OF FRACTIONS ISOLATED FROM ABF (*Didelphis marsupialis*) BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY WITH COOMASSIE BLUE STAIN.
TSK-5PW HPLC-column: lane 1, ABF; lane 2 (F1P); and lane 3 (F2P); Phenyl-Sepharose CL-4B column: lane 4 (F1P); and lane 5 (F2P).

D. marsupialis, *P. opossum* and *L. crassicaudata* to inhibit the lethal action of *B. jararaca* venom has already been described (DOMON *et al.*, 1989; MOUSSATCHÉ *et al.*, 1978, 1990; MOUSSATCHÉ and PERALES, 1989; PERALES *et al.*, 1990).

The present work deals with the tentative quantitative study of the resistance of four South American Didelphidae against *B. jararaca* venom, the isolation of an anti-bothropic fraction from the sera of three of them together with the isolation and partial characterization of an anti-bothropic complex and one of its subunits found in the serum of the opossum *D. marsupialis*.

Of the four marsupials studied, *M. nudicaudatum* was the only one that did not show resistance to the lethal effects of *B. jararaca* venom. Owing to the availability of the species *D. marsupialis* it received the highest doses. A maximum dose of 160 LD_{50} (400 μg/g) was injected i.p. without any noticeable visual effects. As a curiosity, this amount of venom would correspond to an equivalent injection of 24 g of *B. jararaca* venom in a 60 kg adult male/female. *Philander opossum* and *L. crassicaudata* were also resistant to the venom effects. They received lower venom doses since the number of specimens was limited.

The sera of these marsupials were sources for the isolation of an anti-bothropic fraction employing an adaptation of SHIBATA's procedure (SHIBATA *et al.*, 1977) for the purification of acid alpha-1-glycoproteins. In spite of the fact that all four sera had a common fraction eluted at the same sodium chloride concentration upon DEAE-Sephacel chromatography, suggesting physico-chemical properties of alpha-1-glycoproteins, the anti-bothropic activity was found in the peak obtained only from the sera of *Didelphis marsupialis*, *Philander opossum* and *Lutreolina crassicaudata*. The fraction isolated from *Metachirus nudicaudatum* serum was unable to neutralize the lethal action of *B. jararaca* venom.

Approximately the same am unt of protein (300 μg/g) from the serum of any marsupial was needed to neutralize 2 LD_{50} (9.2 μg/g) of the bothropic venom, whereas an effective dose (ED_{50}) of 162 $\pm$ 18 μg was necessary to inhibit the lethality of 1 LD_{50} (4.6 μg/g). The ED_{50} of ABF isolated from *D. marsupialis* serum averaged 13.25 $\pm$ 0.9 μg/g. These results mean that 1 μg of *B. jararaca* venom per g of mice is neutralized by 35 μg of *D. marsupialis* serum proteins and by 2.9 μg of its ABF; an increase in the inhibition of lethality of 12 times.

Electrophoresis of the descending arm of the active fraction from *D. marsupialis* in the presence of SDS showed the existence of only two proteins stained by Coomassie blue (Fig. 2, lane 8) and Schiff's reagent (data not shown). This result, taken together with the HPLC-gel permeation fractionation of the descending arm (Fig. 4A), which demonstrates the presence of only one peak, indicates the existence of an anti-bothropic complex of 84 kDa that can be separated by SDS-PAGE into two subunits of 48 and 43 kDa. These molecular masses are close to those of the most known antineurotoxic and anti-hemorrhagic factors, whose molecular masses range from 52 to 90 kDa (GARCIA and PEREZ, 1984; MENCHACA and PEREZ, 1981; OMORI-SATOH *et al.*, 1972; OVADIA *et al.*, 1977; OVADIA, 1978; PICHYANGKUL and PEREZ, 1981; TOMIHARA *et al.*, 1987, 1988; WEISSENBERG *et al.*, 1991).

This is the first example in the literature of the existence of a complex with anti-bothropic properties. A recent work by WEISSENBERG *et al.* (1991) reports the assumption of the existence of several different antihemorrhagins present in the serum of *Crotalus atrox*. This seems not to be the case among marsupials.

The pure subunit of 48 kDa isolated by low-pressure hydrophobic interaction chromatography (F1P) did not show protective activity in the assay conditions used. The second fraction isolated in this system (F2P) is impoverished in the 48 kDa subunit since this free subunit was isolated from it. This suggests that in F2P there is less 48 kDa subunit available for complex formation with the 43 kDa, which could mean that F2P has a lower amount of active complex than ABF. F2P possess anti-bothropic venom activity. If this is a true reasoning it could explain why a higher amount of F2P (30 μg) is needed to neutralize the lethality of bothropic venom than that needed of ABF (10 μg). In other words, 10 μg of ABF would contain more active complex than 30 μg of F2P. These results also demonstrate that the presence of the subunit of 43 kDa is necessary for the protective action of the complex. It is not known, however, whether the presence of both subunits is needed for activity or whether the 43 kDa subunit can protect mice by itself. If this is the case no explanation can be provided at the moment for the different specific activities of ABF and F2P.

Since no sequences were obtained for the 43 kDa subunit its amino-terminal group might be blocked. It is not known, however, whether this blocking exists in the native protein or is an artifact produced during isolation. No attempts were made to characterize the chemical nature of the blocking group.

N-terminal amino acid sequences of the 48 kDa subunits isolated by SDS-PAGE and Western blotting presented a high degree of homology. Analysis of their sequences indicates the following:

(1) the first five residues are invariant for the three marsupials;

(2) the first 15 residues of *D. marsupialis* and *L. crassicaudata* differ only at position 13, where lysine is exchanged for glutamine. This could imply that they are phylogenetically closer than to *P. opossum*;

(3) proline in positions 6 and 9 in *D. marsupialis* and *L. crassicaudata* is exchanged for threonine and glutamic acid in *P. opossum*. Owing to the special geometry of the prolyl-peptide bond these exchanges imply conformational modifications of the polypeptide backbone at these positions. Thus, it can be supposed that the N-terminal region is not fundamental both for complex formation and for the exercise of protection;

(4) mutations on the first base (K for Q and P for T) and on the first two bases (P for E) are found in this region. In any case, these mutations involve the exchange between purines and pyrimidines.

Even though the number of residues sequenced was small, homology of the amino acid sequence of the 48 kDa subunit to known protein families was searched for on the Protein Identification Resource (release 33.0, PIR, NBRF, U.S.A.) and the Swiss Protein Sequence (release 23.0, Swiss-Prot, Switzerland). No significant homologies were found, indicating that more structural data are needed in order to define whether this subunit belongs to a known protein family.

Acknowledgements—This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq; Financiadora de Estudos e Projetos, FINEP; Coordenação de Aperfeiçoamento do Pessoal de Nivel Superior, CAPES and Fundação de Amparo a Pesquisa do Rio de Janeiro, FAPERJ. We thank Prof. C. R. Diniz, Fundação Ezequiel Dias (FUNED) for the gift of *B. jararaca* venom.

REFERENCES

Andrews, P. (1964) Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91**, 222–233.

Cerdas, L. and Lomonte, B. (1982) Estudio de la capacidad ofiófaga y la resistencia de la zopilota (*Clelia clelia Colubridae*) de Costa Rica a los venenos de serpientes. *Toxicon* **20**, 936–939.

De Witt, C. A. and Weström, B. R. (1987) Venom resistance in the hedgehog (*Erinaceus europaeus*) purification and identification of macroglobulin inhibitors as plasma antihemorrhagic factors. *Toxicon* **25**, 315–323.

Domont, G. B., Perales, J. and Moussatché, H. (1989) Partial purification and physico-chemical characterization of a proteic complex from *Philander opossum* with protective action against *Bothrops jararaca* venom. Brazilian-Sino Symposium on Chemistry and Pharmacology of Natural Products, 10–14 December, Rio de Janeiro, Brazil.

Domont, G. B., Perales, J. and Moussatché, H. (1991) Natural anti-snake venom proteins. *Toxicon* **29**, 1183–1194.

Fontana, F. (1781) Traité sur le venim de la vipére, p. 23. Florence; apud Phisalix, M. (1922) *Animaux Venimeux et Venins*, pp. 744–759. Paris: Mason & Cie Editeurs.

Fortes-Dias, C. L., Diniz, C. R. and Kochva, E. (1990) Neutralization by homologous plasma of *Crotalus durissus terrificus* (South American rattlesnake) venom and crotoxin. *Ciênc. Cult.* **42**, 501–506.

Garcia, V. E. and Perez, J. C. (1984) The purification and characterization of an antihemorrhagic factor in woodrat (*Neotoma micropus*) serum. *Toxicon* **22**, 129–138.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T-4. *Nature* **227**, 608–609.

Landucci, E. C. T., Farah, M. F. L., Giglio, J. R., Domont, G. B. and Marangoni, S. (1990) Protecting property of *Didelphis albiventris* serum against *Bothrops jararaca* snake venom. *Mem. Inst. Butantan* **52** (Suppl.), 76.

Lowry, O. H., Rosebrough, N. J., Farr, L. A. and Randall, R. S. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

Menchaca, J. M. and Perez, J. C. (1981) The purification and characterization of an antihemorrhagic factor in opossum (*Didelphis virginiana*) serum. *Toxicon* **19**, 623–632.

Miranda, M. E., Couturier, G. A., Canziani, A., Seki, C. and Vidal, J. C. (1982) Resistencia de *Crotalus durissus terrificus* y *Bothrops neuwiedii* a la neurotoxicidad de cantidades masivas de veneno crotálico. *Acta Physiol. Latinoamericana* **32**, 103–115.

Moussatché, H. and Perales, J. (1989) Factors underlying the natural resistance of animals against snake venoms. *Mem. Ins. Oswaldo Cruz* **84**, 391–394.

Moussatché, H., Yates, A., Leonardi, F. and Borche, L. (1978) Experimentos sobre la resistencia del Didelphis venezolano (rabi-pelado) a los venenos de serpientes. *Acta Cient. Venezolana* **29**, 55.

MOUSSATCHÉ, H., YATES, A., LEONARDI, F. and BORCHE, L. (1979) Mechanisms f resistance of the opossum to some snake venoms. *Toxicon* **17**, 130.

MOUSSATCHÉ, H. and LEONARDI, F. (1982) Estudios de protección con sueros de mamíferos y reptiles a los venenos de serpientes Crotalidae. *Acta Cient. Venezolana* **33**, 151.

MOUSSATCHÉ, H., PERALES, J., FERREIRA, A. G. C. N., ROCHA, S. L. G., VILLELA, C. G. and DOMONT, G. B. (1990) Resistencia de marsupiais brasileiros aos venenos de *Bothrops jararaca* e *Crotalus durissus terrificus*. VIth Annual Meeting, Federation of Societies of Experimental Biology, MG, Brazil.

OMORI-SATOH, T. (1977) Antihemorrhagic factor as a proteinase inhibitor isolated from the serum of *Trimeresurus flavoviridis*. *Biochim. biophys. Acta* **495**, 93–98.

OMORI-SATOH, T., SADAHIRO, S., OHSAKA, A. and MURATA, R. (1972) Purification and characterization of an antihemorrhagic factor in the serum of *Trimeresurus flavoviridis*, a crotalidae. *Biochim. biophys. Acta* **285**, 414–426.

OVADIA, M. (1978) Purification and characterization of an antihemorrhagic factor from the serum of the snake *Vipera palaestinae*. *Toxicon* **16**, 661–672.

OVADIA, M. and KOCHVA, E. (1977) Neutralization of Viperidae and Elapidae snake venoms by sera of different animals. *Toxicon* **15**, 541–547.

OVADIA, M., MOAV, B. and KOCHVA, E. (1975) Factors in the blood serum of *Vipera palaestinae* neutralizing fractions of its own venom. *Toxicon* **13**, 113.

OVADIA, M., KOCHVA, E. and MOAV, B. (1977) The neutralization mechanism of *Vipera palaestinae* neurotoxin by a purified factor from homologous serum. *Biochim. biophys. Acta* **491**, 370–386.

PERALES, J., MUÑOZ, R. and MOUSSATCHÉ, H. (1986) Isolation and partial characterization of a protein fraction from the opossum (*Didelphis marsupialis*) serum, with protecting property against the *Bothrops jararaca* snake venom. *An. Acad. brasil. Ciênc.* **58**, 155–162.

PERALES, J., MUÑOZ, R., GRATEROL, S., OVIEDO, O. and MOUSSATCHÉ, H. (1989) New findings on the purification and characterization of an anti-bothropic factor from *Didelphis marsupialis* (opossum) serum. *Braz. J. Med. biol. res.* **22**, 25–28.

PERALES, J., DOMONT, G. B., FERREIRA, A. G. C. N., ROCHA, S. L. G. VILLELA, C. G. and MOUSSATCHÉ, H. (1990) Atividade antibotrópica de soros de vários marsupiais brasileiros. *Mem. Inst. Butantan* **52** (Suppl.), 77.

PEREZ, J. C., HAWS, W. C., GARCIA, V. E. and JENNINGS, B. M. (1978*a*) Resistance of warm-blooded animals to snake venoms. *Toxicon* **16**, 375–383.

PEREZ, J. C., HAWS, W. C. and HATCH, H. (1978*b*) Resistance of woodrats (*Neotoma micropus*) to *Crotalus atrox* venom. *Toxicon* **16**, 198–200.

PHILPOT, V. B. and SMITH, R. G. (1950) Neutralization of pit viper venom by KING snake serum. *Proc. Soc. exp. Biol. Med.* **75**, 521–523.

PICHYANGKUL, S. and PEREZ, J. C. (1981) Purification and characterization of a naturally occurring anti-hemorrhagic factor in the serum of the hispid cotton rat (*Sigmodon hispidus*). *Toxicon* **19**, 205–215.

SHIBATA, K., OKUBO, H., ISHIPASHI, H. and TSUDA, K. (1977) Rat alpha$_1$ acid glycoprotein purification and immunological estimation of its serum concentration. *Biochim. biophys. Acta* **495**, 37–45.

TANIZAKI, M. M., KAWAZAKI, H., SUZUKI, K. and MANDELBAUM, F. R. (1991) Purification of a proteinase inhibitor from the plasma of *Bothrops jararaca* (jararaca). *Toxicon* **29**, 673–681.

THOMPSON, W. R. and WEIL, C. S. (1952) On the construction of tables for moving-average interpolation. *Biometrics* **8**, 51–54.

TOMIHARA, Y., YONAHA, K., NOZAKI, M., YAMAKAWA, M., KAMURA, T. and TOYAMA, S. (1987) Purification of three antihemorrhagic factors from the serum of a mongoose (*Herpestes edwardsii*). *Toxicon* **25**, 685–689.

TOMIHARA, Y., YONAHA, K., NOZAKI, M., YAMAKAWA, M., KAWAMURA, Y., KAMURA, T. and TOYAMA, S. (1988) Purification of an anti-haemorrhagic factor from the serum of the non-venomous snake *Dinodon semicarinatus*. *Toxicon* **26**, 420–423.

VELLARD, J. (1945) Resistencia de los 'Didelphis' (Zarigueya) a los venenos ofidicos (Nota previa). *Rev. Bras. Biol.* **5**, 463–467.

WEBER, K. and OSBORN, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate polyacrilamide gel electrophoresis. *J. biol. Chem.* **244**, 4406–4412.

WEISSENBERG, S., OVADIA, M., FLEMINGER, G. and KOCHVA, E. (1991) Antihemorrhagic factors from the blood serum of the western diamondback rattlesnake *Crotalus atrox*. *Toxicon* **29**, 807–818.